DST methods: EU validation & applications at NCCOS/ CCEHBR

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Background:

There are a number of existing LC-MS methods for quantitation of lipophilic marine biotoxins in shellfish that can be grouped according to the LC mobile phases used

- **Acidic (pH ~2)** mobile phases with formic acid and ammonium formate as the additives (*Suzaki et al., Anal. Sci. 27 (2011) 571, McNabb et al., J. AOAC Int. 88 (2005) 761*).
- **Neutral (pH ~6.8)** mobile phases with ammonium acetate as the additive (*Stobo et al., J. AOAC Int. 88 (2005) 1371*).
- **Slightly alkaline (pH ~7.9)** mobile phases with ammonium bicarbonate as the additive (*These et al., J. Chromatogr. A 1216 (2009) 4529*).
- **Alkaline (pH ~11)** mobile phases with ammonium hydroxide as the additive (*Gerssen et al., J. Chromatogr. A 1216 (2009) 1421*).

García-Altares et al, J. Chromatogr. A 1275 (2013) 48

NCCOS/CCEHBR participated in an EU-based inter-laboratory validation study for quantitation of the lipophilic toxins in shellfish using alkaline mobile phases

Inter-laboratory validation study for quantitation of lipophilic marine biotoxins in shellfish using alkaline mobile phases in Sept., 2010

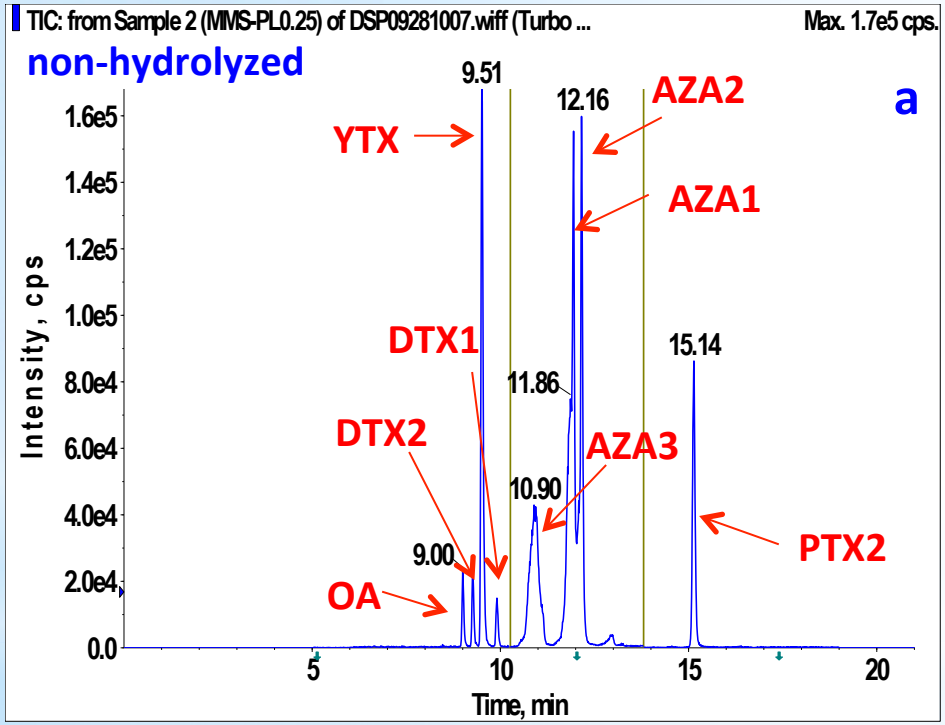
Background:

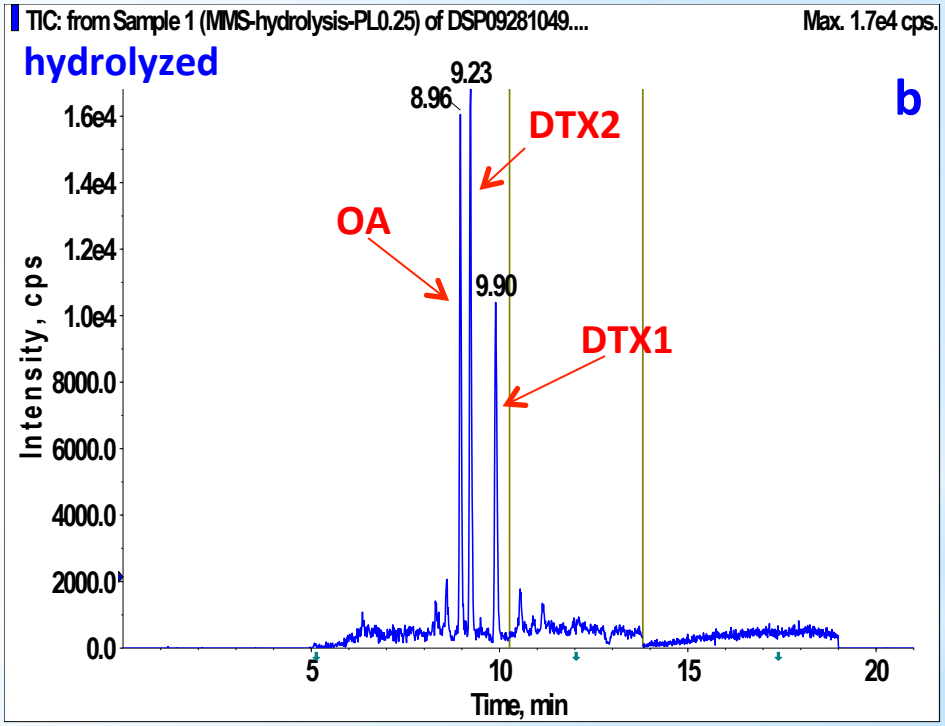
- study coordinated by RIKILT Inst. of Food Safety, The Netherlands
- total of 13 laboratories (12 EU, 1 US) participated in an inter-laboratory study to evaluate method performance characteristics of LC-MS/MS method for lipophilic marine biotoxins
- method evaluated for mussel (*Mytilus edulis*), oyster (*Crassostrea gigas*), and cockle (*Cerastoderma edule*) matrices
- analogues tested: okadaic acid (OA), dinophysistoxins-1 and -2 (DTX1, -2), azaspiracids-1, -2 and -3 (AZA1, -2, -3), pectenotoxin-2 (PTX2), yessotoxin (YTX), and 45-OH-yessotoxin (45-OH-YTX)
- validation conducted according to AOAC-harmonized protocol for design, conduct, and interpretation of method-performance studies

van den Top et al., Food Addit. Contam. Part A, 28 (2011) 1745-1757

Inter-laboratory validation study for quantitation of lipophilic marine biotoxins in shellfish using alkaline mobile phases in Sept., 2010

Mobile phases: 6.7 mM NH_4OH in HPLC water (A) or in 90% acetonitrile (B).
LC column: Waters X-Bridge C18, 150 × 3 mm, 5 μm.
LC gradient: 1 min 10% B, linear gradient to 90% B at 12 min, held for 3 min, returned to 10% B at 17 min and held for 4 min, flow rate 0.4 ml/min.





Figures: Total ion chromatograms of *matrix-matched toxin standards* with concentration of each toxin at 25% (4 ng/ml in an LC vial; except YTX = 25 ng/ml) of permitted level (EU regulatory level: 160 μg/kg) for non-hydrolyzed samples (**a**) and hydrolyzed samples (**b**).

van den Top et al., Food Addit. Contam. Part A, 28 (2011) 1745-1757

Inter-laboratory validation study for quantitation of lipophilic marine biotoxins in shellfish using alkaline mobile phases in Sept., 2010

Data requirements:

- Linearity of calibration curves of ≥ 0.98
- Slope difference < 25% for calibration curves bracketing a sample set
  - ✓ CCEHBR results < 6% for calibration curves bracketing 26 shellfish samples
- Sensitivity of S/N > 6 for the confirmation MRM channel, for calibration standards at 25% of permitted level (4 ng/ml in LC vial with shellfish matrix (YTX=25 ng/ml); 10 μl injection of 1 g shellfish matrix in 10 ml methanol)
- Maximum error in ion ratios of two MRM channels (confirmation:quantitation channels) for matrix-matched standards ≤ 25%
  - ✓ CCEHBR results < 6%
- Maximum relative error in retention shift for matrix-matched standards < 5%
  - ✓ CCEHBR results < 0.4%; EU-RL-MB < 3% (2015)

Inter-laboratory validation study for quantitation of lipophilic marine biotoxins in shellfish using alkaline mobile phases in Sept., 2010

Overall Results:

- recovery values for all participating laboratories were within the range of 80-108% for all toxins except PTX2 (pre-release reference material), which was in the range of 62-93%

- based on the acceptable values for precision and recovery, it was concluded that the method is suitable for official control purposes to quantitatively determine OA/DTXs, AZAs, YTXs, and PTX2 in shellfish

Why do we use alkaline mobile phases at CCEHBR for routine analysis of DSP toxins, PTXs, and AZAs?

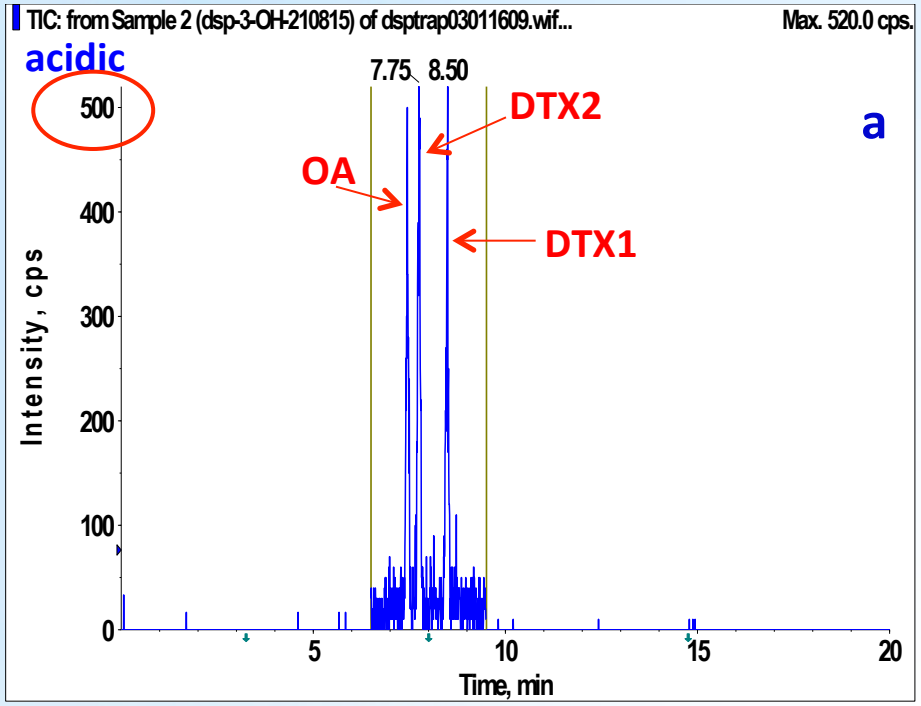
- ***sensitivity*** of DST analysis by LC/MS with alkaline mobile phases is much higher than with acidic mobile phases

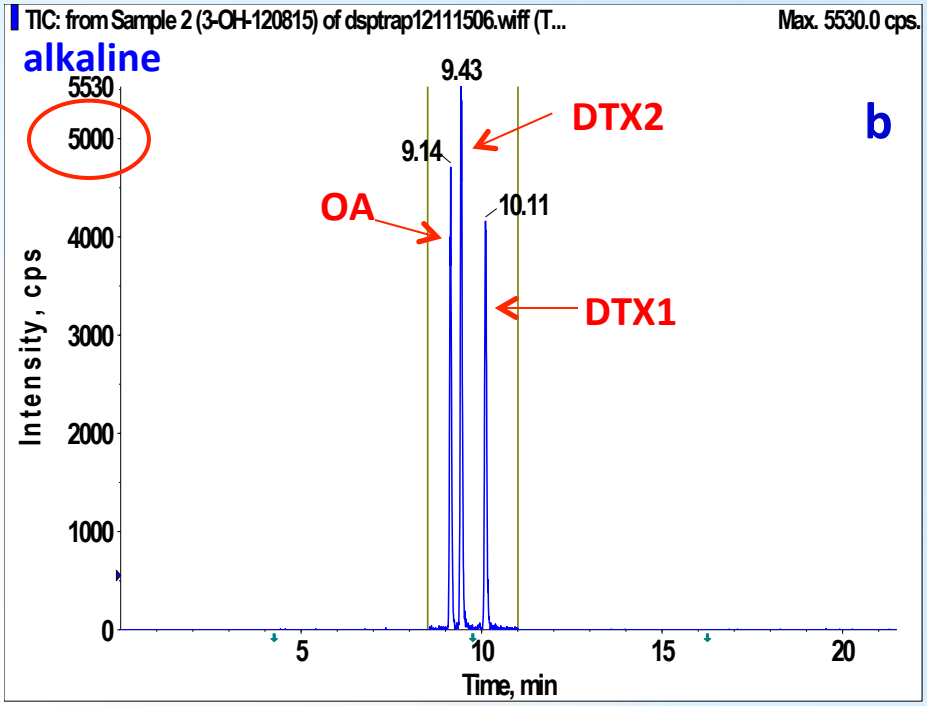
- acidic mobile phases are not practical for use of monitoring DSTs in field-collected algal particulate samples and marine mammal samples
  - a result of poor sensitivity vs. alkaline mobile phase method, when analyzed using moderately sensitive mass spectrometers (e.g., CCEHBR's 4000 QTRAP)

    (Wang et al., J. Chromatogr. A 1416 (2015) 22)

- alkaline mobile phases allow analysis of DSP toxins (non-lipid forms), PTXs, and AZAs in a single LC/MS run with 4000 QTRAP and lower/older version instruments
  - due to the longer time required for switching between positive and negative ion modes
  - higher/newer version instruments are capable of switching over much shorter time intervals

Sensitivity comparison of acidic mobile phases and alkaline mobile phases with HP1100 for separation and 4000 QTRAP (CCEHBR) as a detector





Figures: LC/MS total ion chromatograms of each DSP toxin of 3 ng/ml for an injection volume of 5 μl with a flow rate of 0.4 ml/min.

a: **acidic mobile phases** was 2 mM $HCOONH_4$ and 50 mM HCOOH in HPLC H_2O (A) or in 95% CH_3CN (B). LC column was Phenomenex Luna C8(2), 50 × 2 mm, 5 μm. LC gradient started with 20% B, linear gradient to 80% at 7 min and held for 7 min before return for equilibration.

b: **alkaline mobile phases** was 6.7 mM NH_4OH in HPLC water (A) or in 90% acetonitrile (B). LC column was Waters X-Bridge C18, 150 × 3 mm, 5 μm. LC gradient started with 1.5 min 10% B, linear gradient to 90% at 12.5 min and held for 3 min before return for equilibration.

Overall, alkaline mobile phase generally shows higher sensitivity for DSP toxins regardless of column type and specific shellfish matrix; this trend varies for other lipophilic toxins

Salt effects (0.25 M NaCl) and use of on-line SPE (guard column) with alkaline mobile phases: maintaining MS sensitivity for DSP hydrolyzed samples

failing to eliminate/reduce salt content causes a loss of sensitivity

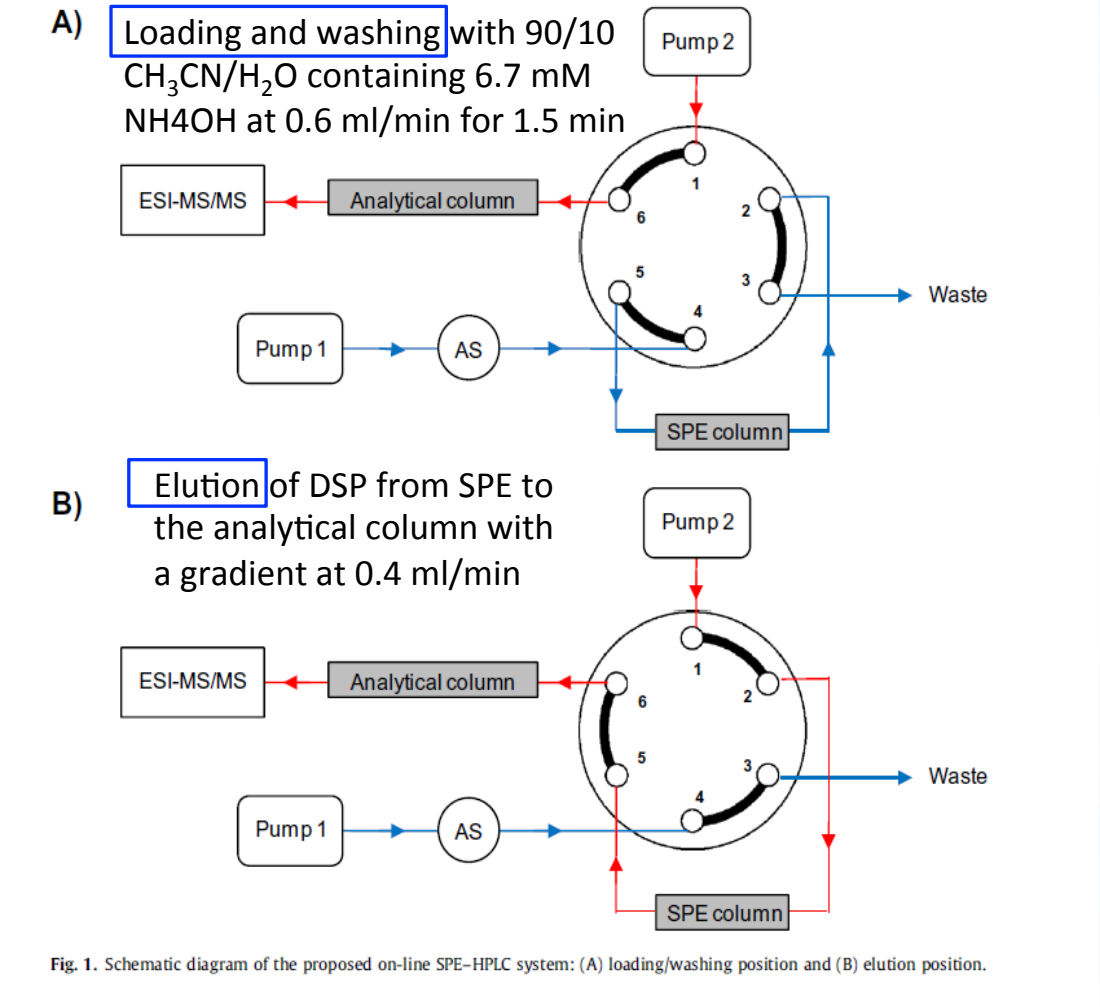


Fig. 1. Schematic diagram of the proposed on-line SPE–HPLC system: (A) loading/washing position and (B) elution position.

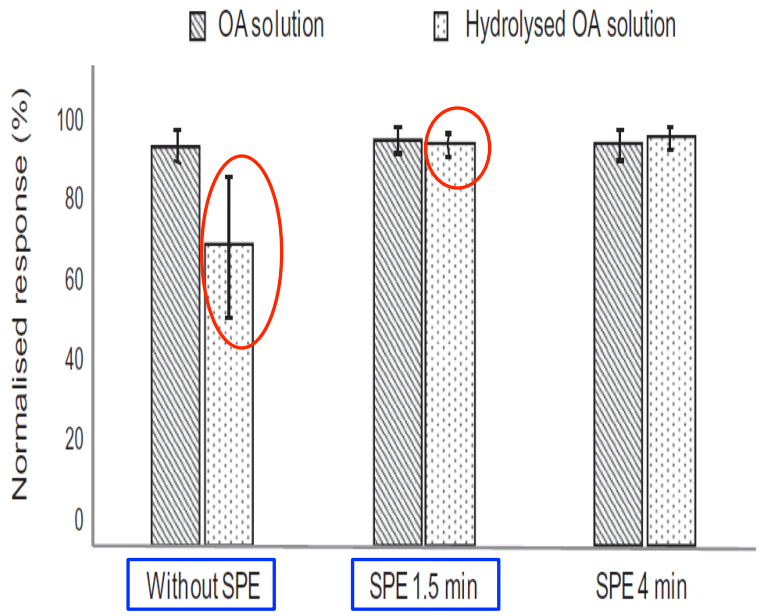


Fig. 2. Normalised responses obtained for a hydrolysed OA solution with and without on-line SPE.

Notes: LC mobile phases for elution were H_2O (A) and CH_3CN/H_2O (90:10, V:V) (B), both containing 6.7 mM NH_4OH. Without on-line SPE, the LC gradient was 25% B for 2.5 min, linear gradient to 95% B over 6 min and held for 2 min before return to initial conditions; ***LC eluate was diverted to waste for the 1st 1.5 min***.

Regueiro et al., Food Chemistry, 129 (2011) 533

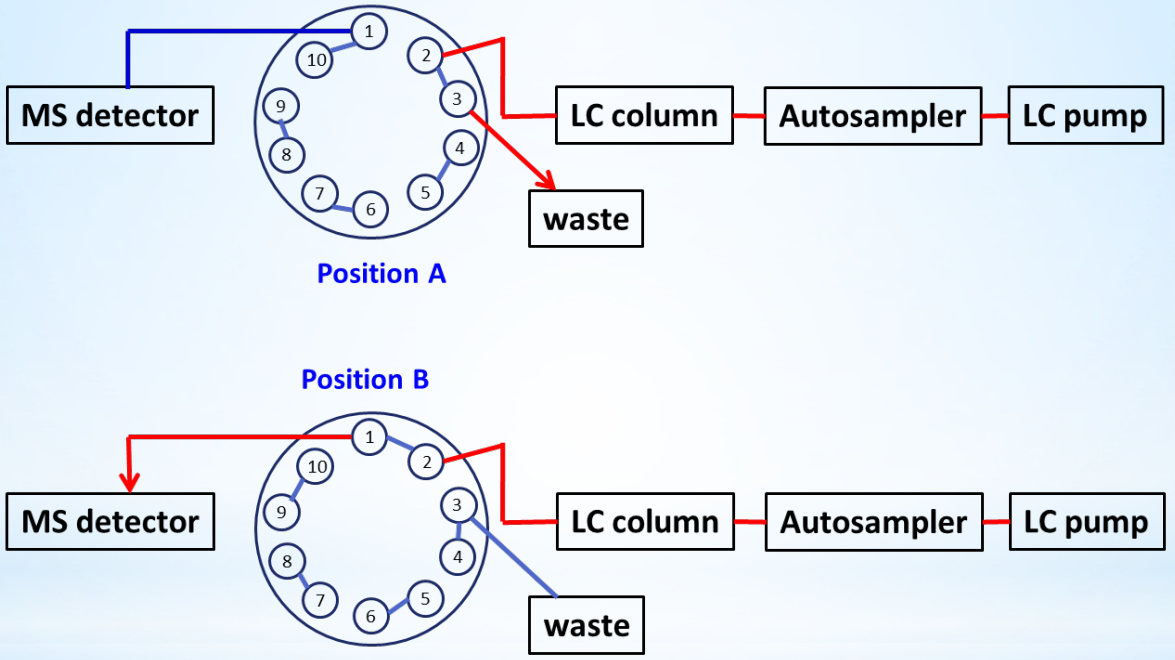
Advantages and disadvantages of on-line SPE method for salt and matrix reduction

Advantage: very efficient at removing salts from hydrolysis process and avoiding loss of MS sensitivity caused by salts

Disadvantage: requires use of two LC pump systems in combination with a diverter valve, which increases the complexity of the instrumentation

CCEHBR strategy for salt removal and MS sensitivity maintenance during LC/MS runs (without use of on-line SPE)

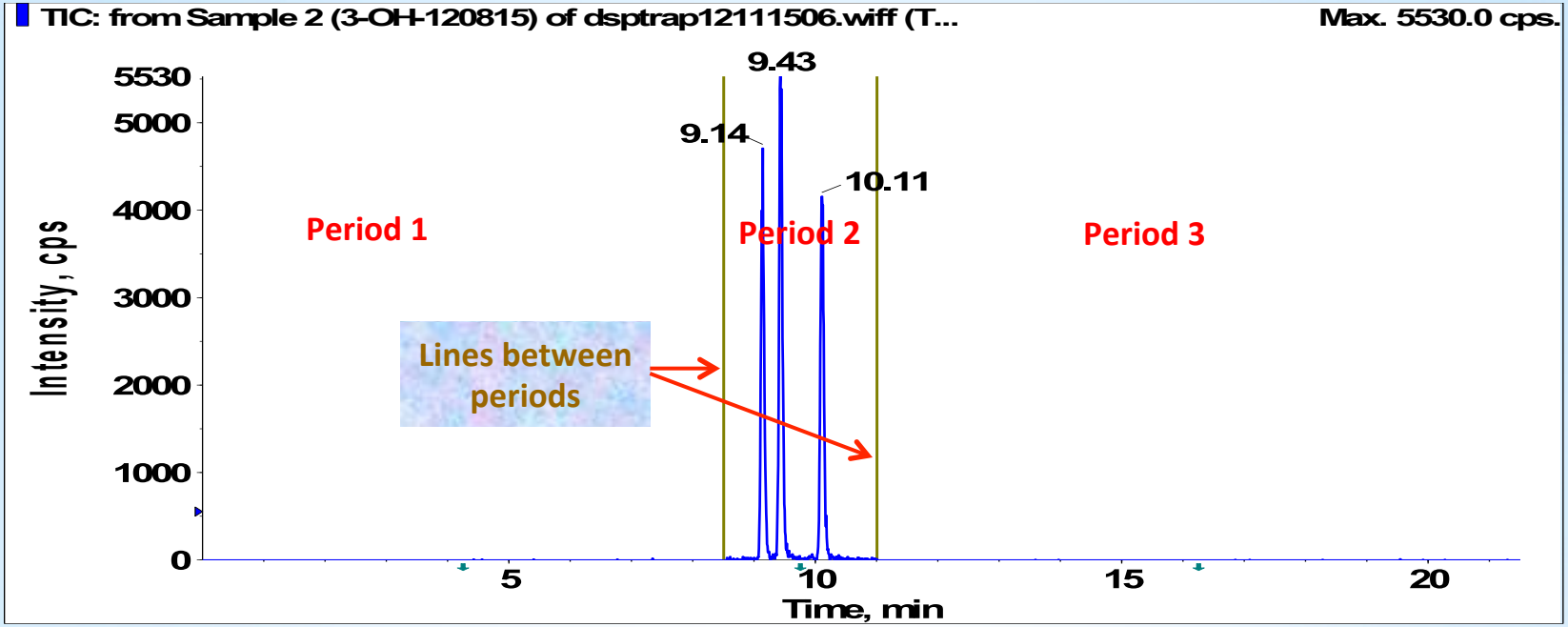
LC settings: one LC pump system is used with a diverter valve; the majority of salts from hydrolyzed samples are removed through LC gradient programming to waste



MS settings: MS scans are divided into periods during an LC run:

- only during periods containing toxins for detection, is the LC eluate is sent to MS
- otherwise, the LC eluate is sent to waste with MS probe voltage set at 0 kV

MS settings for MS sensitivity maintenance during LC/MS runs



Period 1: LC eluant sent to waste using a diverter valve, Turbo Ion Spray probe voltage (IS) set at 0 kV

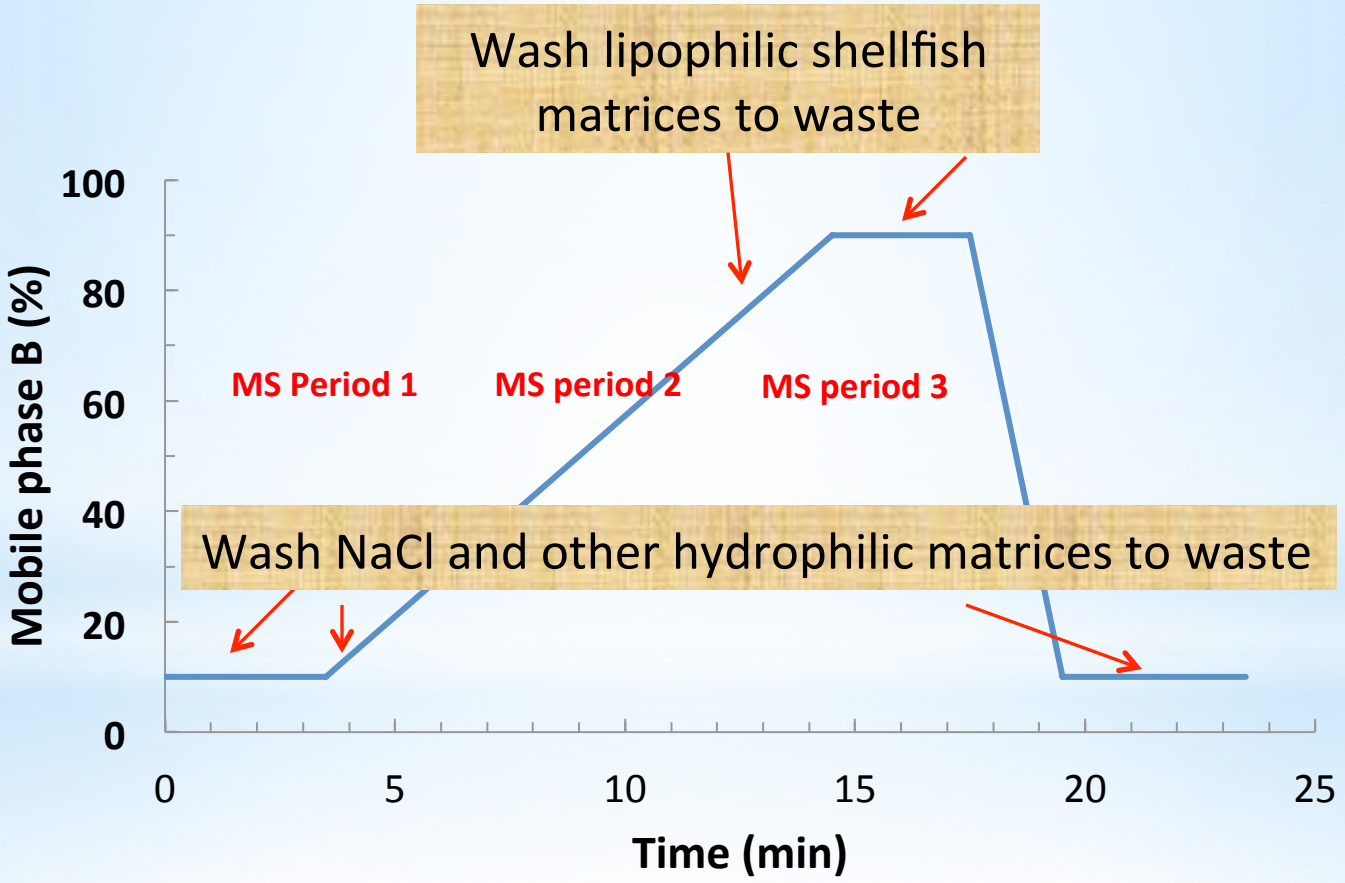
Period 2: LC eluant sent to MS with beginning less than 2 min before 1st toxin peak (OA) and ending less than 2 min after the last toxin peak (DTX1); IS set at -3.5 kV

note: maximum IS voltage is -4.5 kV; a lower value was used to reduce/avoid arcing of TIS probe in negative ion mode

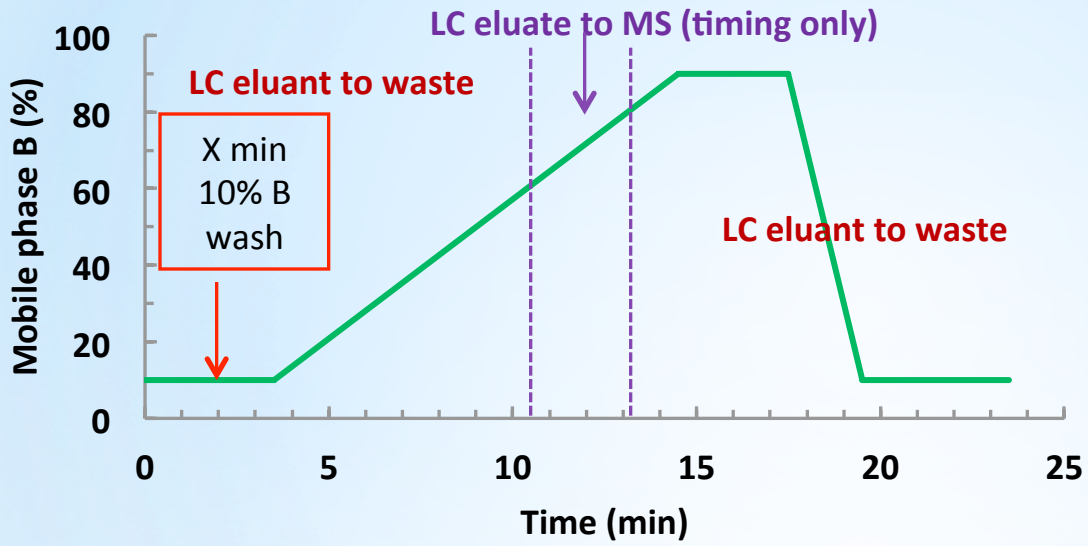
Period 3: same diverter valve and IS settings as Period 1

LC gradient settings for MS sensitivity maintenance during LC/MS runs of hydrolyzed DSP samples

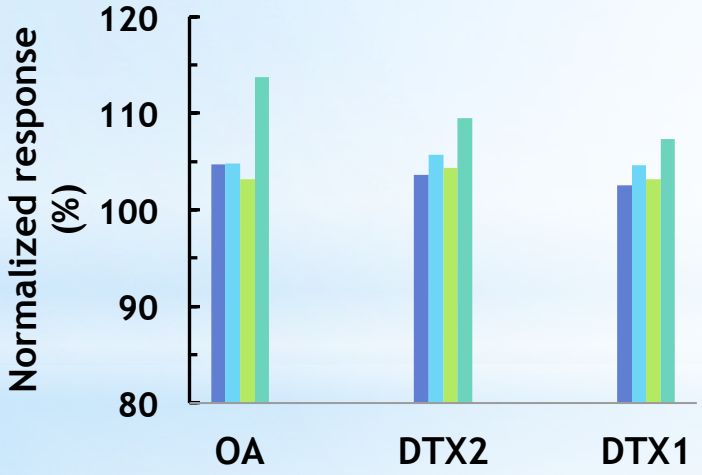
Note: 0.25 M NaCl salt generated for non-concentrated hydrolyzed samples

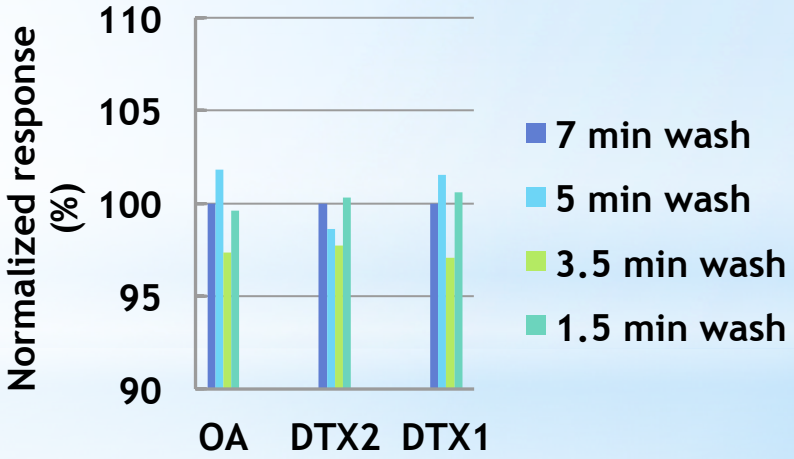


MS responses with different lengths of wash time for salt removal under *alkaline* mobile phase conditions



LC Column: X-Bridge C18; 150 x 3mm
Mobile phases: H_2O (A) and 90% CH_3CN/H_2O (B), both containing 6.7 mM NH_4OH
LC gradients: started with 10% B for a different length of time (e.g., 3.5 min; top figure)
Injection volume: 5 µl
Solutions for injections: one containing 0.25 M NaCl and the other one without NaCl, both containing 3 ng/ml of each DSP toxin





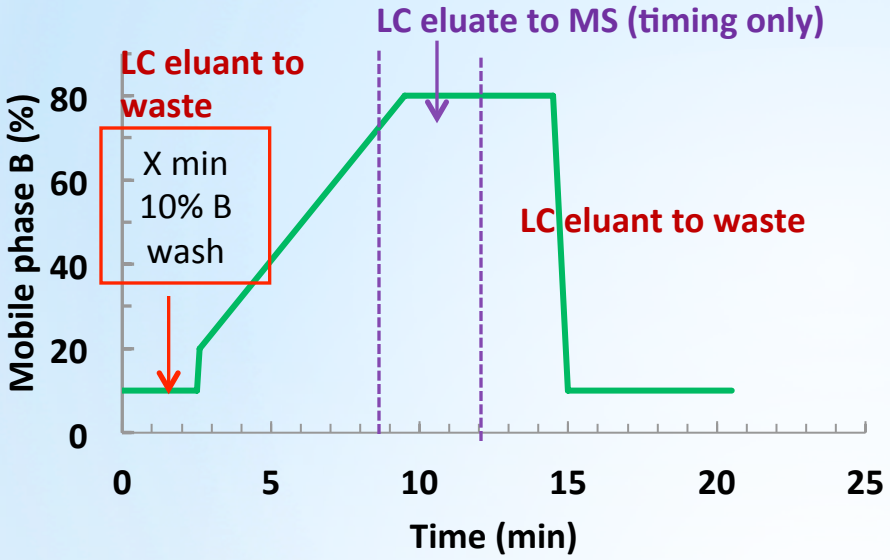
MS responses of DSP toxins *with NaCl normalized to those without NaCl* under the corresponding LC/MS conditions. RSD ≤ 4% (MS responses for each condition with 3 replicates)

**signal enhancement in presence of trace NaCl*

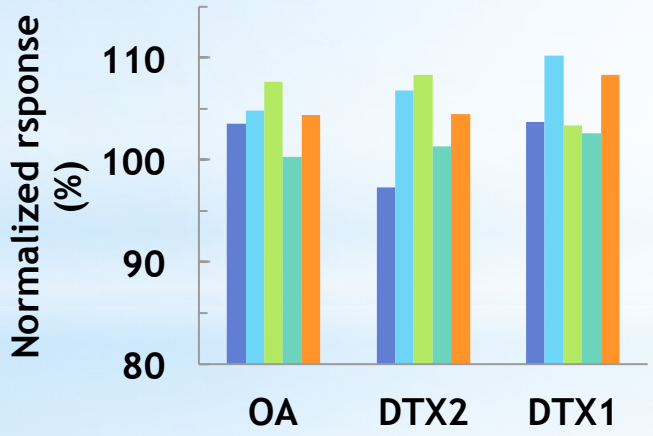
MS responses from the solution *without NaCl were normalized to those LC gradient initiated with maximum wash* (7 min of 10% B)

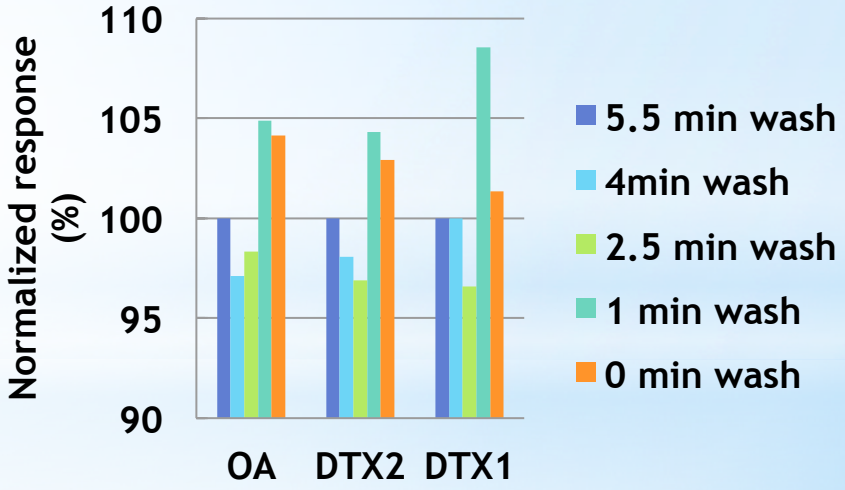
**no change in MS response with length of wash time for toxins in MeOH without NaCl*

MS responses with different lengths of wash time for salt removal under *acidic* mobile phase conditions



LC Column: Luna C8(2); 50 x 2mm
Mobile phases: H_2O (A) and 95% CH_3CN/H_2O (B), both containing 2 mM $HCOONH_4$ and 50 mM HCOOH
LC gradients: started with 10% B for a different length of time (e.g., 2.5 min; top figure)
Injection volume: 10 µl
Solutions for injections: one containing 0.25 M NaCl and the other one without NaCl, both containing 3 ng/ml of each DSP toxin





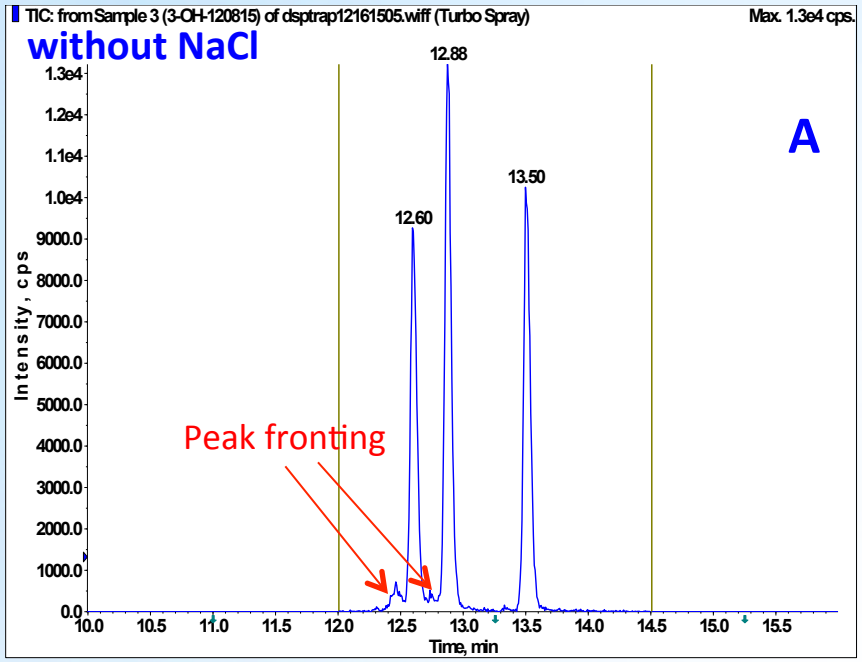
MS responses of DSP toxins *with NaCl normalized to those without NaCl* at corresponding LC/MS conditions. RSD < 8% (MS responses for each condition with 3 replicates)

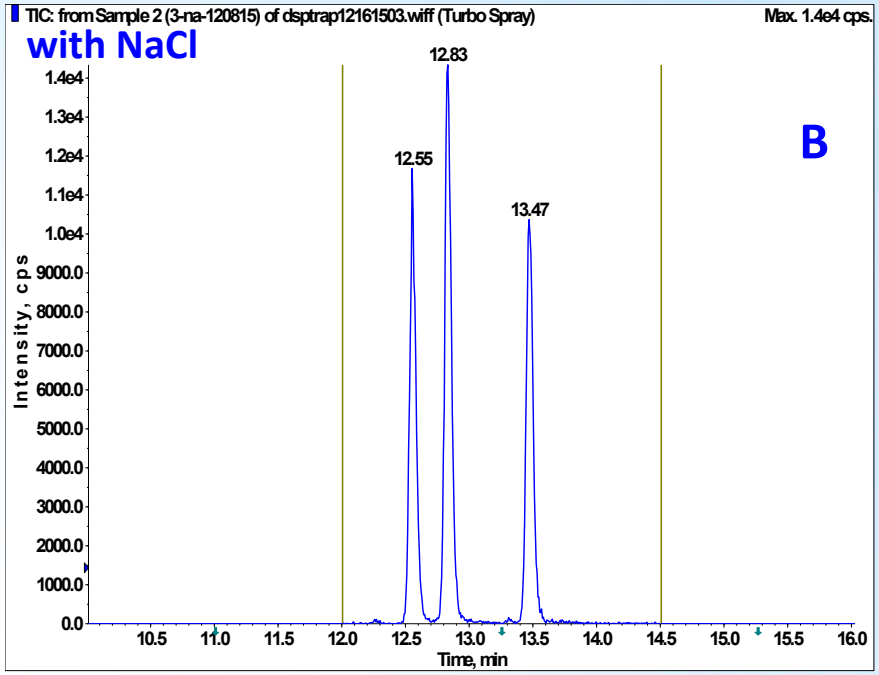
**general enhancement of response with trace NaCl*

MS responses from the solution *without NaCl were normalized to those LC gradient initiated with maximum wash* (5.5 min of 10% B)

**incr. in MS response after running samples with NaCl, likely due to salt build-up on LC/MS interface*

Considerations for injection volumes used with *alkaline* mobile phases





Peak shape comparison of 10 µl injections of DSP solutions without NaCl (**A**) and with 0.25 M NaCl (**B**) when LC gradient initiated with more than 1 min of 10% B

- *10 µl injections can result in poor peak shape with DST standards prepared in MeOH*
- *use of matrix-matched standards eliminates this problem - 10 µl injections used in EU validation study*

No peak shape problems with 5 µl injections or 1 min (or less) of 10% B used to initiate LC gradient with 10 µl injections (note: HPLC water used for mobile phase preparation)

Considerations for adopting external calibration with toxin standards in LC solvents instead of shellfish extracts (matrix-matched)

- matrix issues have been widely reported for the analysis of lipophilic toxins by LC/MS/MS
- matrix-matched standards proven very effective as long as matrix used to prepare standards is consistent with the actual samples being tested (*McCarron et al., J. AOAC Int. 97 (2014) 316)*
- for standards prepared *without* sample matrices, the following strategies may required for quantitation:

    1. removal of matrices by sample preparation or by on-line SPE and LC/MS settings (e.g., salt/matrix removal covered in previous slides)
    2. reduction of sample matrix loading on the analytical LC column (5 μl or less instead of 10 μl injections; sample dilution prior to injection)
    3. separation of matrices from toxins through choice of LC column (ratio of column length to particle size; higher ratio = increased resolution) and LC elution programming
    4. quantitation using external standards *without* matrices *BUT* corrected with recovery rates determined for spiked samples of the same shellfish species/matrix

Next steps at CCEHBR for analysis of lipophilic marine biotoxins in shellfish by LC/MS/MS

1. *Matrix removal through sample preparation:* incorporate hexane wash (easy to use) has no effect on the quantitation, but provides cleaner solutions for maintaining a higher level of instrument performance *(Kilcoyne et al., J. Chromatogr. A 1217 (2010) 7123)*

2. *LC/MS settings:* e.g., periodic diversion to waste, to remove majority of salts & reduce MS interface contamination from salts and shellfish tissue matrices in samples

3. *Separation of interfering matrices from toxins through LC elution programming:* both alkaline mobile phase method *(Gerssen et al. J. Chromatogr. A 1216 (2009) 1421; X-Bridge C18 column)* and acidic mobile phase method *(method from WA Department of Health; Luna C8(2) column)* will be examined for different shellfish matrices
   - quantitation of spike-recovery samples using standards prepared in methanol to determine correction factor (focus on hydrolyzed samples)

4. *Collaboration with partners for inter-laboratory comparison of methods*

Thank you for your attention – Questions?